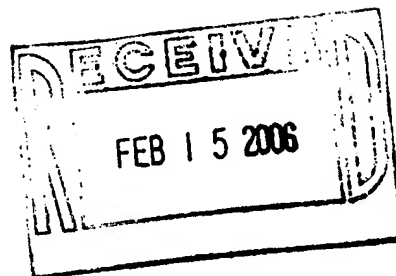


IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.: WARF-0015



Inventors: Anderson et al.

Serial No.: 10/606,038

Filing Date: June 25, 2003

Examiner: Gitomer, Ralph J.

Customer No.: 26259

Group Art Unit: 1655

Confirmation No.: 7983

Title: Methods of Preventing or Treating Cell-Migration Mediated Conditions or Diseases

"Express Mail" Label No. EV688860900US
Date of Deposit: March 6, 2006

I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner for Patents, Mail Stop Non-Fee Amendment, P. O. Box 1450, Alexandria, VA 22313-1450.

By Jane Massey Licata
Typed Name: Jane Massey Licata, Reg. No. 32,257

Commissioner for Patents
Mail Stop Non-Fee Amendment
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

DECLARATION UNDER RULE § 1.131

I, Richard A. Anderson, hereby declare that:

1. I am a co-inventor in U.S. Patent Application Serial No. 10/606,038 filed June 25, 2003 and am most familiar with the subject matter of this application and the research effort which

Attorney Docket No.: **WARF-0015**
Inventors: **Anderson et al.**
Serial No.: **10/606,038**
Filing Date: **June 25, 2003**
Page 2

lead to the discovery of the instant invention. All the work described in the following paragraph occurred at the University of Wisconsin located in Madison, Wisconsin, USA.

2. I have reviewed Caenepeel (US 2005/0125852 A1). This reference is not relevant to the above-referenced application. My invention relates to the discovery that the phosphatidylinositol phosphate kinase Y661 isoform (PIPKIY661) is specifically targeted to focal adhesions by an association with talin. We found that PIPKIY661 was tyrosine phosphorylated by focal adhesion-associated kinase signaling, increasing both the activity of phosphatidylinositol phosphate kinase and its association with talin. This is in contrast to the teachings of Caenepeel, wherein mammalian protein kinases and protein kinase-like enzymes are described. Phosphatidylinositol phosphate kinases and protein kinases have different and distinct substrates, i.e., lipids versus proteins, and are therefore different enzymes.

3. In any event, as evidenced by the publication entitled "Type 1Y Phosphatidylinositol Phosphate Kinase Targets and Regulates Focal Adhesions" ((November 2002) *Nature* 420:89-93) attached hereto, experiments were being carried out in my laboratory prior to May 9, 2003, which led to the assay disclosed in the above-referenced application. For example, we demonstrated binding of talin with PIPKIY661 in the presence of an antibody during immunoprecipitation experiments (see page 90, third full paragraph of column 1). While the antibody employed did not appear to modulate binding of talin to PIPKIY661, this discovery showed

Attorney Docket No.: **WARF-0015**
Inventors: **Anderson et al.**
Serial No.: **10/606,038**
Filing Date: **June 25, 2003**
Page 3

that we could readily detect agents which modulate the interaction between talin and PIPKIY661.

Attorney Docket No.: **WARF-0015**
Inventors: **Anderson et al.**
Serial No.: **10/606,038**
Filing Date: **June 25, 2003**
Page 4

4. The experiments presented in the above-referenced publication occurred prior to May 9, 2003, as evidenced by a reception date of June 2, 2002 for the manuscript by the journal Nature.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

2/11/06
Date



Richard A. Anderson



(see Supplementary Information). Full-length talin 2 clones were PCR-amplified from human brain and skeletal muscle Marathon-Ready cDNA libraries (BD Biosciences Clontech).

Antibodies and constructs

A rabbit serum specific for PtdInsPKI γ -90 was raised against a peptide corresponding to the 28aa-tail (amino acids 635–662) of mouse PtdInsPKI γ -90. The following antibodies were from commercial sources: anti-talin clone 8d4 (Sigma), antiserum C-20 (Santa Cruz Biotechnology) and clone TA205 (Chemicon); anti-vinculin (Sigma) and anti-FAK C-20 (Santa Cruz Biotechnology). Other antibodies used in this study are described elsewhere²⁴. The C-terminal domains of human PtdInsPKI γ -87 (amino acids 459–640) and PtdInsPKI γ -90 (amino acids 458–668) were PCR-amplified from clone KIAA0589 and subcloned into pGEX-6P.1 vector (Amersham-Pharmacia). Deletion constructs were generated in the same vector. For expression of GFP fusion proteins, human PtdInsPKI γ -87, -90, 28aa-tail (amino acids 641–668) and PtdInsPKI γ -90 C-terminal (amino acids 384–668) were subcloned into the pEGFP2 vector (BD Biosciences Clontech). The head of human talin 2 and mouse talin 1, together with fragments from the FERM domain of talin 2, were subcloned into pGEX4T1. Recombinant PtdInsPKI γ -90 was produced in insect cells using the baculovirus system (BD Pharmingen).

Biochemical procedures

Affinity chromatography experiments and immunoprecipitations were performed from rat brain detergent extract as described previously²⁵. For the lipid kinase assay, immunoprecipitates were incubated for 10 min at 37°C in 40 μ M kinase buffer (25 mM HEPES pH 7.4, 100 mM KCl, 1 mM EGTA, 2.5 mM MgCl₂) containing 20 μ M phosphoinositides (Sigma), 20 μ M [γ -³²P]ATP and 50 μ M cold ATP. Samples were then processed as previously described²⁶. For kinase assays involving recombinant PtdInsPKI γ -90, 25 ng enzyme was used. Kinase assays performed with fibroblasts used 50 μ g extract. The blot overlay assay with GST and GST-28aa-tail was performed as described²⁷. The phage display library screening was performed according to ref. 30.

Cell transfection and immunofluorescence

NIH 3T3 cells were grown and maintained on plastic culture plates at 37°C in a 5% CO₂ atmosphere in DMEM containing 10% fetal bovine serum. Cells were detached with PBS supplemented with 0.05% trypsin, 1 mM EDTA, replated on fibronectin-coated glass coverslips and transfected using Lipofectamine reagent (Life technologies). Twenty-four hours later, cells were fixed, immunostained and analysed by either confocal or epifluorescence microscopy. For the quantification of spread cells, 150–200 cells were scored from each of three independent experiments.

Received 12 June; accepted 4 September 2002; doi:10.1038/nature01147.

- De Camilli, P., Emr, S. D., McPherson, P. S. & Novick, P. Phosphoinositides as regulators in membrane traffic. *Science* 271, 1533–1539 (1996).
- Hurley, J. H. & Meyer, T. Subcellular targeting by membrane lipids. *Curr. Opin. Cell Biol.* 13, 146–152 (2001).
- Simonsen, A., Wurmser, A. E., Emr, S. D. & Stenmark, H. The role of phosphoinositides in membrane transport. *Curr. Opin. Cell Biol.* 13, 485–492 (2001).
- Takemura, T. & Itoh, T. Phosphoinositides, key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane. *Biochim. Biophys. Acta* 1533, 190–206 (2001).
- Ishihara, H. et al. Type I phosphatidylinositol 4-phosphate 5-kinases. *J. Biol. Chem.* 273, 8741–8748 (1998).
- Wenk, M. R. et al. PIP kinase γ is the major PI(4,5)P₂ synthesizing enzyme at the synapse. *Neuron* 32, 79–88 (2001).
- Crichtley, D. R. Focal adhesions—the cytoskeletal connection. *Curr. Opin. Cell Biol.* 12, 133–139 (2000).
- Martin, T. F. Phosphoinositide lipids as signalling molecules: common themes for signal transduction, cytoskeletal regulation, and membrane trafficking. *Annu. Rev. Cell Dev. Biol.* 14, 231–264 (1998).
- Sechi, A. S. & Wehland, J. The actin cytoskeleton and plasma membrane connection: PtdIns(4,5)P₂ influences cytoskeletal protein activity at the plasma membrane. *J. Cell Sci.* 113, 3683–3695 (2000).
- Cremers, O. et al. Essential role of phosphoinositide metabolism in synaptic vesicle recycling. *Cell* 99, 179–188 (1999).
- Anderson, R. A., Boronnikov, L. V., Doughman, S. D., Kunz, J. & Lofgren, J. C. Phosphatidylinositol phosphate kinases, a multifaceted family of signaling enzymes. *J. Biol. Chem.* 274, 9907–9910 (1999).
- McPherson, P. S. et al. A presynaptic inositol 5-phosphatase. *Nature* 379, 353–357 (1996).
- Chung, J. K. et al. Synaptotagmin inhibition of phospholipase D activity by hydrolysis of phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* 272, 15960–15965 (1997).
- Gad, H. et al. Fusion and uncoupling of synaptic clathrin-coated vesicles are perturbed by disruption of interactions with the SH3 domain of endophilin. *Neuron* 27, 301–312 (2000).
- Rees, D. J., Ailes, S. E., Singer, S. J. & Hynes, R. O. Sequence and domain structure of talin. *Nature* 347, 685–689 (1990).
- Hanada, K., Shimizu, T., Matsui, T., Tsukita, S. & Hakoshima, T. Structural basis of the membrane-targeting and unmasking mechanisms of the radixin FERM domain. *EMBO J.* 19, 4449–4462 (2000).
- Pearson, M. A., Recek, D., Breischer, A. & Karpus, P. A. Structure of the ERN1 protein moosein reveals the FERM domain fold masked by an extended actin binding tail domain. *Cell* 101, 259–270 (2000).
- Calderwood, D. A. et al. The phosphotyrosine binding-like domain of talin activates integrins. *J. Biol. Chem.* 277, 21749–21758 (2002).
- Fridde, J. et al. Disruption of the talin gene compromises focal adhesion assembly in undifferentiated but not differentiated embryonic stem cells. *J. Cell Biol.* 142, 1121–1133 (1998).
- Chavis, P. & Westbrook, G. Integrins mediate functional pre- and postsynaptic maturation at a hippocampal synapse. *Nature* 411, 317–321 (2001).
- Serra-Pages, C. et al. The LAR transmembrane protein tyrosine phosphatase and a coiled-coil LAR-interacting protein co-localize at focal adhesions. *EMBO J.* 14, 2827–2838 (1995).

- Meriläinen, J., Lehto, V. P. & Wäsänen, V. M. FAP52, a novel, SH3 domain-containing focal adhesion protein. *J. Biol. Chem.* 272, 23278–23284 (1997).
- Zhen, M. & Jin, Y. The liprin protein SYD-2 regulates the differentiation of presynaptic termini in *C. elegans*. *Nature* 401, 371–375 (1999).
- Monkley, S. J., Pritchard, C. A. & Crichtley, D. R. Analysis of the mammalian talin2 gene TLN2. *Biochem. Biophys. Res. Commun.* 286, 880–885 (2001).
- Martel, V. et al. Conformation, localization, and integrin binding of talin depend on its interaction with phosphoinositides. *J. Biol. Chem.* 276, 21217–21227 (2001).
- McNamee, H. P., Ingber, D. E. & Schwartz, M. A. Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol lipid breakdown. *J. Cell Biol.* 121, 673–678 (1993).
- Kunz, J. et al. The activation loop of phosphatidylinositol phosphate kinases determines signaling specificity. *Mol. Cell* 5, 1–11 (2000).
- Tollas, K. F. et al. Type I alpha phosphatidylinositol 4-phosphate 5-kinase mediates Rac-dependent actin assembly. *Curr. Biol.* 10, 153–156 (2000).
- McPherson, P. S. et al. Interaction of Grb2 via its Src homology 3 domains with synaptic proteins including synapsin I. *Proc. Natl. Acad. Sci. USA* 91, 6486–6490 (1994).
- Felici, F., Castagnoli, L., Musacchio, A., Iappelli, R. & Cesareni, G. Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. *J. Mol. Biol.* 212, 301–310 (1991).

Supplementary Information accompanies the paper on Nature's website (<http://www.nature.com/nature>).

Acknowledgements We thank L. Liu, L. Daniell and M. Caleo for technical help. We also thank T. Nagase for the gift of the human PtdInsPKI γ -90 clone KIAA0589; C. Carpenter for the gift of the PtdInsPKI α and β cDNAs; R. Hynes for the gift of mouse talin 1 cDNA; G. Cesareni and F. Felici for the phage library; and laboratory members and O. Cremona for critical discussion. This work was supported in part by grants from the NIH and from the American Diabetes Association to P.D.C. L.P. and G.C. were supported by postdoctoral fellowships from Telethon-Italia, and K.L. is a Howard Hughes Medical Institute predoctoral fellow. S.C. is a Howard Hughes Medical Institute fellow of the Life Sciences Research foundation.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to P.D.C. (e-mail: pietro.decamilli@yale.edu).

Type I γ phosphatidylinositol phosphate kinase targets and regulates focal adhesions

Kun Ling¹*, Renee L. Doughman¹*, Ari J. Firestone², Matthew W. Bunce¹ & Richard A. Anderson¹

¹Department of Pharmacology, Program in Molecular and Cellular Pharmacology, University of Wisconsin-Madison, 1300 University Avenue, University of Wisconsin Medical School, Madison, Wisconsin 53706, USA

*These authors contributed equally to this work.

The ability of cells to form cell contacts, adhere to the extracellular matrix, change morphology, and migrate is essential for development, wound healing, metastasis, cell survival and the immune response. These events depend on the binding of integrin to the extracellular matrix, and assembly of focal adhesions, which are complexes comprising scaffolding and signalling proteins organized by adhesion to the extracellular matrix^{1–3}. Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) regulates interactions between these proteins, including the interaction of vinculin with actin and talin^{4–9}. The binding of talin to β -integrin is strengthened by PtdIns(4,5)P₂, suggesting that the basis of focal adhesion assembly is regulated by this lipid mediator¹⁰. Here we show that the type I phosphatidylinositol phosphate kinase isoform- γ 661 (PIPKI γ 661), an enzyme that makes PtdIns(4,5)P₂, is targeted to focal adhesions by an association with talin. PIPKI γ 661 is tyrosine phosphorylated by focal adhesion-associated kinase signalling, increasing both the activity of phosphatidylinositol phosphate kinase and its

letters to nature

association with talin. This defines a mechanism for spatial generation of $\text{PtdIns}(4,5)\text{P}_2$ at focal adhesions.

Adherence to extracellular matrix stimulates the generation of $\text{PtdIns}(4,5)\text{P}_2$ (refs 4, 6) and potentially modulates focal adhesion assembly around clusters of integrins¹⁻¹⁰. Type I phosphatidylinositol phosphate kinase isoforms (PIPKIs) generate the lipid messenger $\text{PtdIns}(4,5)\text{P}_2$ (refs 11, 12). The PIPKI γ transcripts are alternatively messenger RNA spliced, resulting in the PIPKI γ 635 and PIPKI γ 661 isoforms that differ by a 26-amino-acid carboxy-terminal extension¹³.

PIPKI γ 661 is specifically targeted to focal adhesions (Fig. 1a). To determine if the C-terminal 26 amino acids are sufficient for targeting, these residues were fused to green fluorescent protein, GFP. The GFP γ 26 is poorly targeted to focal adhesions, suggesting that other regions within PIPKI γ 661 are also required. To define these constraints, we prepared chimaeras using PIPKI α , an enzyme not targeted to focal adhesions (see Supplementary Information). The PIPKI α/γ chimaeras contained 178 residues from the C terminus of PIPKI γ 661 (PIPKI α/γ 661) or simply the PIPKI γ 661 26 amino acids fused to the C terminus of PIPKI α (PIPKI α/γ 26). PIPKI α/γ 661 and PIPKI α/γ 26 target to focal adhesions, but not PIPKI α/γ 635 (Fig. 1a). Enhanced targeting of the PIPKI α constructs with the C-terminal 26 residues reflects structural or functional features of the phosphatidylinositol phosphate kinases, such as dimer formation, which is required for activity at membranes^{12,14}.

We used two approaches to identify proteins that specifically interact with PIPKI γ 661. Haemagglutinin (HA)-tagged PIPKI γ 661 or PIPKI γ 635 were expressed in HEK 293T cells, and isolated by immunoprecipitation. As numerous focal adhesion proteins are tyrosine phosphorylated, the immunoprecipitates were immunoblotted with anti-phosphotyrosine antibodies. A tyrosine-phosphorylated protein of relative molecular mass 230,000 (M_r 230K) was selectively immunoprecipitated with PIPKI γ 661 (Supplementary Information). This protein was identified as talin^{15,16} (Fig. 1b). Vinculin, focal adhesion associated kinase (FAK) and tensin did not interact with PIPKI γ 661 (Supplementary Information). Only the PIPKI α/γ chimaeras that target to focal adhesions associated with talin (Fig. 1b). To confirm the talin-PIPKI γ 661 interaction, talin was immunoprecipitated from HEK 293T cells overexpressing PIPKI γ 661 or PIPKI γ 635. Talin pulled down PIPKI γ 661, but not PIPKI γ 635 (Fig. 1b). In addition, endogenous PIPKI γ splice isoforms are widely expressed in various cell lines. When endogenous PIPKI γ was immunoprecipitated from a variety of cells types, talin association was observed (Supplementary Information).

A yeast two-hybrid screen using the 178-residue C-terminal region of PIPKI γ 661 as bait resulted in the isolation of 12 talin clones. This demonstrated a direct interaction, and narrowed the interacting region to residues 150 to 480 in the talin head region. This region encompasses a portion of F1 and the F2 and F3 regions of talin's band 4.1, ezrin, radixin, moesin (FERM) homology domain. The talin FERM domain also contains regions shown to interact with other focal adhesion proteins¹⁷⁻²⁰ (Fig. 1c).

These data show that the PIPKI γ 661-talin association is important for targeting PIPKI γ 661 to focal adhesions. This is reinforced by the immunofluorescent colocalization of PIPKI γ 661 and talin in NRK cells (Fig. 2). As $\text{PtdIns}(4,5)\text{P}_2$ modulates the association of talin with β -integrin and other focal adhesion proteins^{7,9,10}, the PIPKI γ 661-talin interaction may regulate the focal adhesion assembly of talin. Moderate overexpression of PIPKI γ 661 resulted in substantially larger talin-containing focal adhesions in 43% of cells compared to non-transfected cells (Fig. 2). This supports a model where PIPKI γ 661 modulates talin assembly into focal adhesions. High overexpression of PIPKI γ 661, but not PIPKI γ 635, resulted in a loss of talin from focal adhesions in 49% of the transfected cells (Fig. 2). These data indicate that increasing levels of $\text{PtdIns}(4,5)\text{P}_2$ at focal adhesions cause talin assembly and disassembly, suggesting a dynamic model. Overexpression of

PIPKI isoforms reorganizes actin from stress fibres to cortical actin filaments^{11,13}. Thus, phenotypes observed by high overexpression of PIPKI γ 661 may also affect focal adhesions indirectly.

Kinase inactive mutants, when expressed in cells, will often disrupt the function of the wild-type kinase by competing for

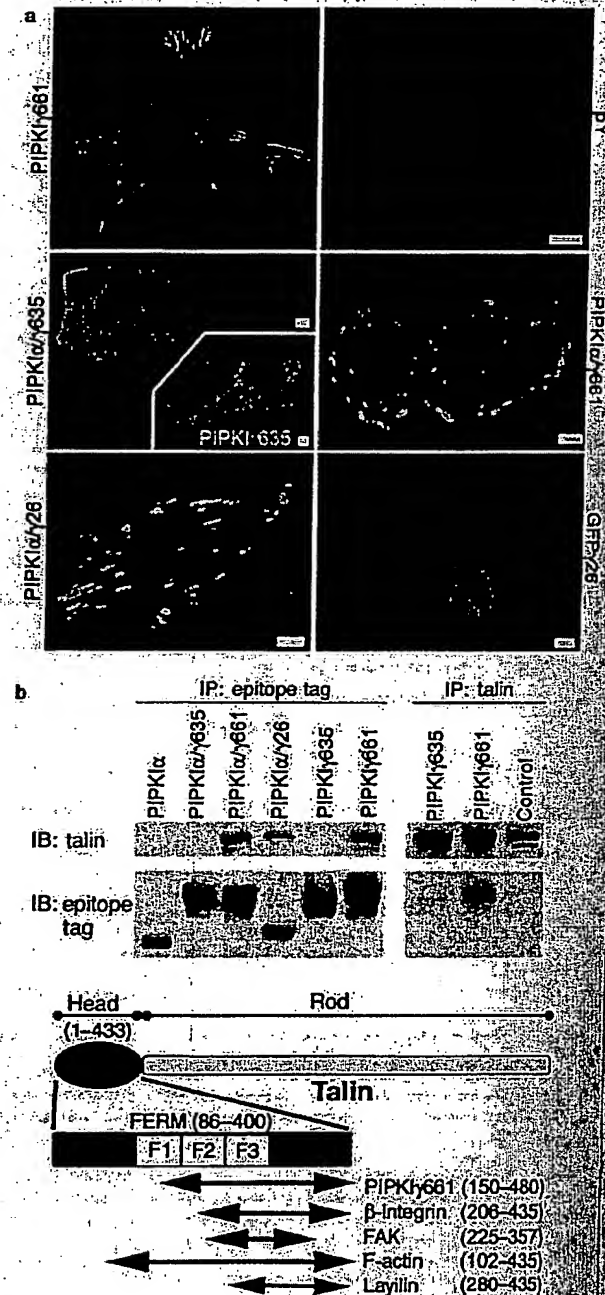


Figure 1 PIPKI γ 661 is targeted to focal adhesions by an association with talin. **a**, Colocalization of HA-PIPKI γ 661 and focal adhesions in NRK cells stained with anti-HA (green) and polyclonal anti-phosphotyrosine (PY) (red) antibodies. Fusion proteins containing the C-terminal 26 amino acids from PIPKI γ 661 show targeting to focal adhesions in NRK cells. **c**, Myc-PIPKI α/γ chimaeras and HA-PIPKI γ 635 were stained with c-Myc and HA antibodies, respectively. See Supplementary Information for generation of chimaeras. Scale bar, 10 μm . **b**, Overexpressed PIPKI constructs were immunoprecipitated (IP) from HEK 293T cells and then immunoblotted (IB) with indicated antibodies. **c**, PIPKI γ 661 interacts with talin in the FERM domain as determined by yeast two-hybrid screen. Illustration of other talin binding proteins in FERM domain.

targeting or regulation. Expression of a kinase-inactive ('kinase-dead') PIPK γ 661 mutant (PIPK γ 661kd) resulted in a loss of talin targeting to focal adhesions in 70% of the cells expressing high levels (Fig. 2). This is consistent with the proposed role for PtdIns(4,5)P $_2$ in talin assembly into focal adhesions¹⁻³.

During adhesion and spreading, cells assemble focal adhesions rapidly, providing a good model to define the role of PIPK γ 661 in membrane and focal adhesion assembly of talin. PIPK γ 661 facilitated the targeting of talin to the plasma membrane in adhering cells (Fig. 3). This was independent of PIP kinase activity, as PIPK γ 661kd also facilitated targeting (Fig. 3 inset; see also Supplementary Information). Significantly, PIPK γ 661 facilitated the assembly of talin-containing focal adhesions in spreading cells, and this was dependent on PIP kinase activity (Fig. 3). These data show that PIPK γ 661 regulates both plasma membrane targeting and the efficient assembly of talin into focal adhesions.

The association between PIPK γ 661 and talin was also regulated by adhesion to type I collagen. In Fig. 4a, we show co-immunoprecipitation of talin with PIPK γ 661 from HEK 293T cells in suspension versus cells adherent to type I collagen. Talin strongly associated with PIPK γ 661 in adherent cells, but not in suspended cells. However, in suspended cells the PIPK γ 661 association with talin was restored when cells were stimulated with lysophosphatidic acid (LPA) or platelet-derived growth factor (PDGF).

Tyrosine phosphorylation of focal adhesion proteins is stimu-

lated by adhesion, and is important for focal adhesion assembly²¹. PIPK γ 661 is tyrosine phosphorylated, and this was dependent upon PIP kinase activity (Fig. 4a). Tyrosine phosphorylation was also stimulated upon cell adhesion to type I collagen. FAK is an important tyrosine kinase at focal adhesions and is activated by LPA²². So to determine FAK's role in tyrosine phosphorylation of PIPK γ 661, we coexpressed dominant active FAK (CD2FAK)²³ with each of the PIPK γ isoforms. This induced a ~4-fold increase in tyrosine phosphorylation of PIPK γ 661, but not PIPK γ 635, illustrating that focal adhesion targeting is required for tyrosine phosphorylation of PIPK γ 661. Tyrosine phosphorylation of PIPK γ 661kd was also stimulated by coexpression of CD2FAK, although to a lesser extent. Coexpression of the dominant-negative FAK related non-kinase (FRNK), which blocks endogenous FAK activity, diminished tyrosine phosphorylation of PIPK γ 661. FRNK also inhibited adhesion-stimulated PIPK γ 661 tyrosine phosphorylation. The FAK-induced tyrosine phosphorylation of PIPK γ 661 correlates with a substantial increase in its association with talin. Tyrosine phosphorylation of PIPK γ 661 may facilitate association with talin via the phosphotyrosine-binding site in the talin head²⁰. Significantly, CD2FAK but not FRNK specifically stimulated the activity of PIPK γ 661. These data indicate that FAK signalling stimulates both talin association and PIP kinase activity, resulting in localized generation of PtdIns(4,5)P $_2$ at focal adhesions (see Supplementary Information).

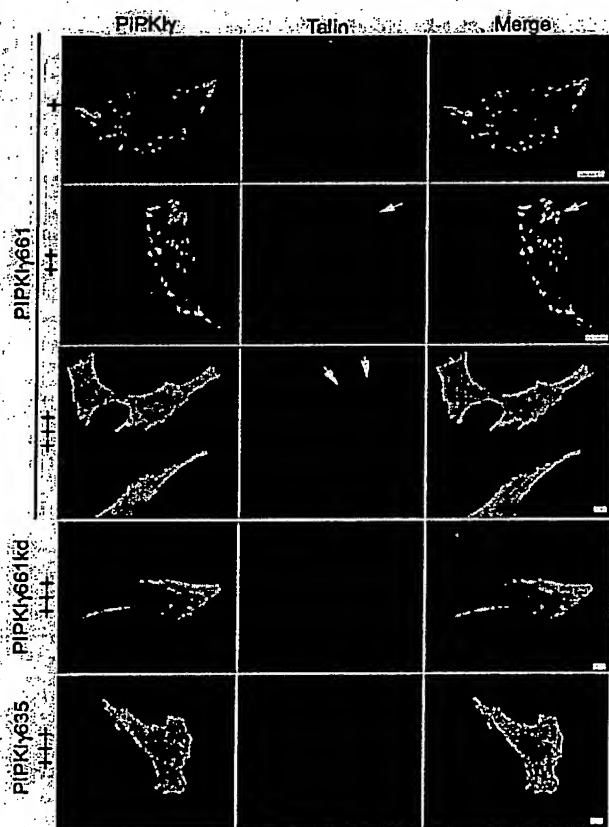


Figure 2 PIPK γ 661 modulates assembly of talin into focal adhesions. Overexpressed PIPK γ and endogenous talin in NRK cells were visualized by anti-PIPK γ serum (green) and anti-talin antibody (red), respectively. Relative expression level of PIPK γ is shown (+). Low HA-PIPK γ 661 (+) colocalized with talin, and the increasing moderate expression (++) induced larger talin-containing focal adhesions, whereas high overexpression (+++) diminished talin assembly into focal adhesions. High expression of PIPK γ 661kd abolished talin assembly into focal adhesions. High expression of PIPK γ 635 constructs had no effect on focal adhesions. Scale bar, 10 μ m.

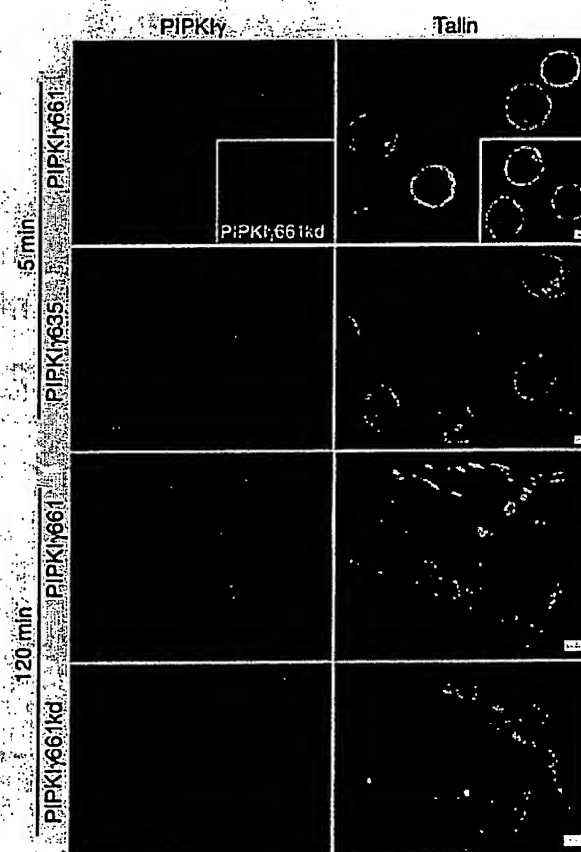


Figure 3 PIPK γ 661 facilitates talin targeting to membranes and assembly into focal adhesions. Transfected NRK cells were suspended for 1 h, then replated on type I collagen (see Methods). After the indicated time, localization of PIPK γ (green) and talin (red) in cells was visualized with anti-PIPK γ serum and anti-talin antibodies, respectively. Inset, location of PIPK γ 661kd and talin. See Supplementary Information for quantification of PIPK γ and talin localization. Scale bar, 10 μ m.

A functional link between PIPKI-661 and FAK was reinforced by the observation that PIPKI-661kd but not PIPKI-661 disrupts targeting of FAK to focal adhesions. PIPKI-661 and PIPKI-661kd both target to focal adhesions and colocalize with FAK (Fig. 4b and

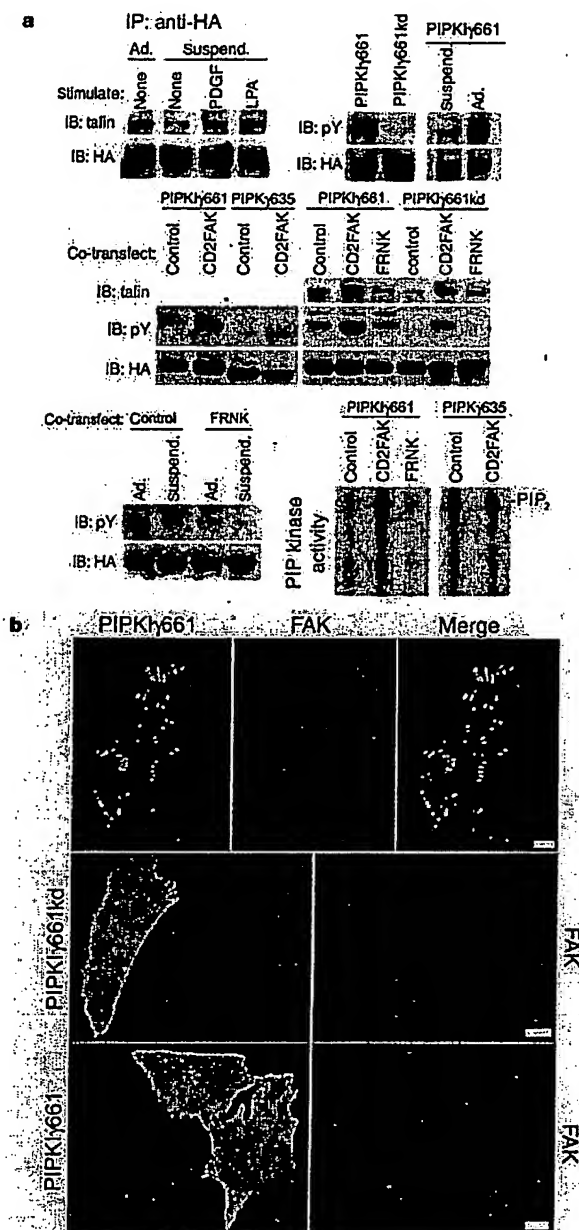


Figure 4 Functional linkage between PIPKI-661 and FAK signalling. **a**, In all experiments, HA-PIPKI-661 was immunoprecipitated (IP) from HEK 293T cells with anti-HA antibody and analysed by immunoblotting (IB) or kinase assay as indicated. Top left, PIPKI-661–talin association in adherent (ad.), suspended (suspend.), or suspended cells stimulated as indicated. Top right, PIPKI-661, but not PIPKI-661kd, is tyrosine phosphorylated, which is dependent upon adhesion. Middle, phosphorylation of PIPKI-661 and talin association were shown when cotransfected with CD2FAK or FRNK. Bottom left, adhesion-dependent PIPKI-661 phosphorylation was inhibited by FRNK. Bottom right, PIPKI-661 activity when coexpressed with indicated proteins. (See Supplementary Information for quantification.) **b**, High expression of PIPKI-661kd but not PIPKI-661 (visualized by anti-PIPKI-661 serum, green) abolishes FAK (visualized with anti-FAK, red) from focal adhesions in NRK cells. Scale bar, 10 μ m. $N \geq 4$ for all results in this Letter.

data not shown). High expression of PIPKI-661 did not affect FAK targeting. In contrast, the expression of PIPKI-661kd resulted in complete loss of FAK targeting to focal adhesions in 39% of the expressing cells. A number of other focal adhesion proteins were analysed to determine the effect of PIPKI-661 and PIPKI-661kd expression. From these studies, paxillin and vinculin were more resistant to changes induced by ectopic expression of PIPKI-661 or PIPKI-661kd (see Supplementary Information).

The interaction between PIPKI-661 and talin appears critical for focal adhesion assembly. This is supported by observations that a β -integrin binding site regulated by PtdIns(4,5) P_2 is located in the head region of talin within the FERM domain^{9,17–19}. Proteins containing the FERM domain, such as protein 4.1 and talin, interact with integral membrane proteins, such as glycophorin or integrins, and these interactions are modulated by PtdIns(4,5) P_2 (refs 9, 19, 24, 25). Therefore, the PIPKI-661–talin interaction may be important in the initiation of focal adhesion assembly via regulation of integrin binding and other focal adhesion proteins by PtdIns(4,5) P_2 (refs 1–3, 7–9).

We suggest that, upon clustering of integrins, talin and PIPKI-661 are recruited to focal adhesions, inducing synthesis of PtdIns(4,5) P_2 . Spatial generation of PtdIns(4,5) P_2 facilitates the recruitment and regulation of proteins such as vinculin, α -actinin and FAK. FAK, in a manner that depends on PtdIns(4,5) P_2 , tyrosine phosphorylates PIPKI-661, and further stimulates production of PtdIns(4,5) P_2 . Regulated and localized generation of PtdIns(4,5) P_2 facilitates the assembly and/or disassembly of focal adhesions. PIPKI activity is also regulated by small G proteins^{14,26} and by phosphatidic acid, a product of phospholipase D (PLD)²⁷. PLD activity is required for actin stress fibre formation and α -actinin assembly at focal adhesions²⁸. In many cells, adhesion is essential for survival and growth, which require phosphoinositide 3-kinase (PI3K) activity²¹. PI3K is also modulated by FAK, and may be dependent upon PIPKI-661 for its substrate. Consequently, generation of PtdIns(4,5) P_2 by PIPKI-661 may also control the generation of messengers derived from PtdIns(4,5) P_2 . This positions PIPKI-661 at a signalling branch point that modulates both focal adhesion assembly and signals emanating from focal adhesions.

Methods

Constructs

Site-directed mutagenesis for creation of kinase-dead PIPKI-661 (D253A) was performed using PCR-primer overlap extension with mutagenic primers²⁹. The mutagenic primers were 5'-CAAAATGCAGCTTAAGTT-CGCCCTCAAGGGCTCCAC-3' and 5'-GTACGTG GAGCCCTTGAGGGCGAA GTTAACCTGC-3'. The flanking primers were 3'-CTATG CAGCTGTTCCTTCGGTACTTC-3' and 5'-GGCGGTGGAATACAGAGCCCTTC-3'. The mutation was confirmed by DNA sequence analysis. A 0.5-kilobase *Xba*I–*Glu*I restriction fragment was used to replace the corresponding restriction fragment in the HA-PIPKI-661/pCDNA3 construct. See Supplementary Information for generation of GFP constructs, c-Myc-PIPKI-661 chimaeras, and recombinant protein expression vectors.

Yeast two-hybrid screen

The yeast two-hybrid screen³⁰ was performed in the Molecular Interaction Facility at the University of Wisconsin according to their protocols. Libraries screened were mouse embryonic, human B cell, human breast, human prostate, human placenta and mouse brain. See <http://www.biotech.wisc.edu/mif/Lipid>.

Kinase assays

Activity of purified recombinant or immunoprecipitated PIPKI proteins was assayed against 25 μ M PIP micelles as previously described³¹.

Cell culture, transfection, immunofluorescence and confocal microscopy

HEK 293T cells and NRK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc.) supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics. NRK cells were transfected using FuGENE 6 (Roche) according to manufacturer's instructions for 24 h. HEK 293T cells in 1 μ g ml⁻¹ collagen-coated dishes were transfected using 2 μ g PIP kinase expression vector together with 3 μ g empty pCDNA3 vector, CD2FAK, or FRNK as indicated, using the calcium phosphate–DNA coprecipitation method for 48 h.

Immunofluorescence and confocal microscopy were performed as described³². Z series were created by sequentially scanning green and red channels at 0.2 μ m steps. Single sections were exported to Adobe Photoshop 6.0 for final image processing.

Expression and purification of PIP kinase in *Escherichia coli*

His-tagged PIPK α , PIPK β 661 and C-tail of PIPK β 661 were expressed in *E. coli* and purified as described²².

Antibodies

Anti-talin, anti-vinculin and anti-Flag (M2) antibodies are from Sigma. Monoclonal mouse anti-PY (4G10), anti-FAK (4.47) and anti-paxillin (5H11) are from Upstate. Polyclonal rabbit anti-PY antibody is from Transduction Laboratories. Anti-HA and anti-c-Myc antibodies are from Covance. Polyclonal PIPK β anti-serum was generated by Covance using purified His-tagged PIPK β 661. Secondary antibodies are from Jackson ImmunoResearch. Anti-serum was purified on an affinity column generated by coupling recombinant C-terminus of PIPK β 661 to cyanogen bromide-activated Sepharose 4B (Sigma) as described²³.

Immunoprecipitation and immunoblotting

Immunoprecipitation was performed as described²⁴. Briefly, transfected HEK 293T cells were harvested and lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 5.0 mM NaF, 2 mM Na₂VO₄, 4 mM Na₂P₂O₇, 1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10% glycerol, then centrifuged and incubated with protein A-Sepharose and 5 μ g anti-HA, anti-c-Myc, anti-Flag, or anti-talin antibody as indicated at 4°C overnight. The immunocomplexes were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and analysed as indicated. For kinase assay, the immunocomplexes were washed by and stored in kinase buffer.

Cell stimulation and spreading

Transfected HEK 293T cells were washed with PBS and detached with 0.25% trypsin at 37°C. The cells were harvested and washed with PBS, and then suspended in serum-free DMEM at 37°C for 1 h. After stimulation with 10 ng ml⁻¹ platelet-derived growth factor (PDGF) (R&D Systems Inc.) or 1 μ M LPA (Sigma) or serum at 37°C for 15 min, cells were lysed as above for immunoprecipitation. For spreading assays, transfected NRK cells were detached with 1 mM EDTA, washed with PBS, then 0.1 \times 10⁶ cells were plated on 1 μ g ml⁻¹ collagen-coated coverslips in serum-free DMEM. After the indicated time, the coverslips were fixed and stained as described above.

Received 2 June; accepted 1 August 2002; doi:10.1038/nature01082.

1. Gatchley, D. R. Focal adhesions—the cytoskeleton connection. *Curr. Opin. Cell Biol.* **12**, 133–139 (2000).
2. Burridge, K. & Chrzanowska-Wodnicka, M. Focal adhesions, contractility, and signalling. *Annu. Rev. Cell Dev. Biol.* **12**, 463–518 (1996).
3. Schwartz, M. A. & Ginsberg, M. H. Networks and cross-talk: integrin signalling spreads. *Nature Cell Biol.* **4**, E65–E68 (2002).
4. McNamee, H. P., Ingber, D. E. & Schwartz, M. A. Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol lipid breakdown. *J. Cell Biol.* **121**, 673–678 (1993).
5. Berditchevski, F., Tolia, K. F., Wong, K., Carpenter, C. L. & Hemler, M. E. A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. *J. Biol. Chem.* **272**, 2595–2598 (1997).
6. Chong, L. D., Traynor-Kaplan, A., Bokoch, G. M. & Schwartz, M. A. The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell* **79**, 507–513 (1994).
7. Gilmose, A. P. & Burridge, K. Regulation of vinculin binding to talin and actin by phosphatidylinositol-4,5-bisphosphate. *Nature* **381**, 531–535 (1996).
8. Steinle, P. A., Hoffer, J. D., Adey, N. B. & Craig, S. W. Polyphosphoinositides inhibit the interaction of vinculin with actin filaments. *J. Biol. Chem.* **274**, 18414–18420 (1999).
9. Martel, V. et al. Conformation, localization, and integrin binding of talin depend on its interaction with phosphoinositides. *J. Biol. Chem.* **276**, 21217–21227 (2001).
10. Janney, P. A. Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. *Annu. Rev. Physiol.* **56**, 163–191 (1994).
11. Anderson, R. A., Boronnikov, I. V., Doughman, S. D., Kim, J. & Lojens, J. C. Phosphatidylinositol phosphate kinases, a multifaceted family of signaling enzymes. *J. Biol. Chem.* **274**, 9907–9910 (1999).
12. Kim, J. et al. The activation loop of phosphatidylinositol phosphate kinases determines signaling specificity. *Mol. Cell* **5**, 1–11 (2000).
13. Ishihara, H. et al. Type I phosphatidylinositol-4-phosphate 5-kinases. Cloning of the third isoform and deletion/substitution analysis of members of this novel lipid kinase family. *J. Biol. Chem.* **273**, 8741–8748 (1998).
14. Rao, V. D., Misra, S., Boronnikov, I. V., Anderson, R. A. & Hurley, J. H. Structure of type II β phosphatidylinositol phosphate kinase: a protein kinase fold flattened for interfacial phosphorylation. *Cell* **94**, 829–839 (1998).
15. Pasquale, E. B., Maher, P. A. & Singer, S. J. Talin is phosphorylated on tyrosine in chicken embryo fibroblasts transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA* **83**, 5507–5511 (1986).
16. DeCloe, J. E. & Martin, G. S. Phosphorylation of talin at tyrosine in Rous sarcoma virus-transformed cells. *Mol. Cell Biol.* **7**, 371–378 (1987).
17. Calderwood, D. A. et al. The Talin head domain binds to Integrin β subunit cytoplasmic tails and regulates integrin activation. *J. Biol. Chem.* **274**, 28071–28074 (1999).
18. Rees, D. J., Ailes, S. E., Singer, S. J. & Hynes, R. O. Sequence and domain structure of talin. *Nature* **347**, 685–689 (1990).
19. Chisti, A. H. et al. The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. *Trends Biochem. Sci.* **23**, 281–282 (1998).
20. Calderwood, D. A. et al. The phosphotyrosine binding (PTB)-like domain of talin activates integrins. *J. Biol. Chem.* **277**, 21749–21758 (2002).
21. Cary, L. A. & Guan, J. L. Focal adhesion kinase in integrin-mediated signaling. *Front. Biosci.* **4**, D102–D113 (1999).
22. Rodriguez-Fernandez, I. L. & Rozengurt, E. Bombesin, vasopressin, lysophosphatidic acid, and sphingosylphosphorylcholine induce focal adhesion kinase activation in intact Swiss 3T3 cells. *J. Biol. Chem.* **273**, 19321–19328 (1998).

23. Chan, P. Y., Kanner, S. B., Whitney, G. & Aruffo, A. A transmembrane-anchored chimeric focal adhesion kinase is constitutively activated and phosphorylated at tyrosine residues identical to pp125FAK. *J. Biol. Chem.* **269**, 20567–20574 (1994).
24. Anderson, R. A. & Marchesi, V. T. Regulation of the association of membrane skeletal protein 4.1 with glycophorin by a polyphosphoinositide. *Nature* **318**, 295–298 (1985).
25. Hira, M. et al. Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. *J. Cell Biol.* **133**, 37–51 (1996).
26. Honda, A. et al. Phosphatidylinositol 4-phosphate 5-kinase α is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* **99**, 521–532 (1999).
27. Jenkins, G. H., Fiset, P. L. & Anderson, R. A. Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. *J. Biol. Chem.* **269**, 11547–11554 (1994).
28. Kim, Y. & Eton, J. H. Phospholipase D activity is required for actin stress fiber formation in fibroblasts. *Mol. Cell Biol.* **21**, 4055–4066 (2001).
29. James, P., Halladay, J. & Craig, E. A. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144**, 1425–1436 (1996).
30. Lojens, J. C. & Anderson, R. A. Type I phosphatidylinositol-4-phosphate 5-kinase are distinct members of this novel lipid kinase family. *J. Biol. Chem.* **271**, 32937–32943 (1996).
31. Zhang, X. L. et al. Phosphatidylinositol-4-phosphate 5-kinase isoforms catalyze the synthesis of 3-phosphate-containing phosphatidylinositol signaling molecules. *J. Biol. Chem.* **272**, 17756–17761 (1997).

Supplementary information accompanies the paper on Nature's website (<http://www.nature.com/nature>).

Acknowledgements HA-tagged PIPK β mammalian expression vectors were a gift of T. F. J. Martin. We thank D. Mosher, A. Huttenlocher and P. Keely for discussions and reagents. This research was supported by the National Institutes of Health R.A.A., and by the American Heart Association for K.L., R.L.D. and M.W.B.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to R.A.A. (e-mail: raanders@facstaff.wisc.edu).

Nbs1 is essential for DNA repair by homologous recombination in higher vertebrate cells

Hiroshi Tauchi*, Junya Kobayashi†, Ken-ichi Morishima†, Dik C. van Gent‡, Takahiro Shirahishi†, Nicole S. Verkalk†, Diana van Heemst†, Emi Ito†, Asako Nakamura†, Eiichi Sonoda§, Minoru Takata||, Shunichi Takeda§, Shinya Matsuura† & Kenshi Komatsu†¶

* Department of Environmental Sciences, Faculty of Science, Ibaraki University, Bunkyo 2-1-1, Mito, Ibaraki 310-8512 Japan

† Department of Radiation Biology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan

‡ Department of Cell Biology and Genetics, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam, The Netherlands

§ Department of Radiation Genetics, Faculty of Medicine, ¶ Radiation Biology Research Center, Kyoto University, Yoshida Konoe, Sakyo-ku, Kyoto 606-8501 Japan

|| Immunology and Molecular Genetics, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan

Double-strand breaks occur during DNA replication and are also induced by ionizing radiation. There are at least two pathways which can repair such breaks: non-homologous end joining and homologous recombination (HR). Although these pathways are essentially independent of one another, it is possible that the proteins Mre11, Rad50 and Xrs2 are involved in both pathways in *Saccharomyces cerevisiae*. In vertebrate cells, little is known about the exact function of the Mre11–Rad50–Nbs1 complex in the repair of double-strand breaks because Mre11- and Rad50-